Optimization of Embryonic Stem Cell Proliferation and Differentiation for High Throughput Screening of Embryotoxic Drugs and Chemicals

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Bachelor of Science with Honors Research Distinction in The Ohio State University

By

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2021

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Abstract

Many chemicals present in food, drug, and the environment may be embryotoxic and can cause embryo malformation. Embryonic stem cell test (EST) is a novel in vitro method to detect the embryotoxicity of chemicals. However, current EST based on cardiac differentiation is limited to its long assay time and complex cell culture and morphological assessment of cardiomyocytes. Moreover, current EST cannot evaluate drug effects on stem cell differentiation to other cell lineages and suffers from low predictability of <80 percent. The goal of our research is to develop a high throughput embryotoxicity screening based on enhanced green fluorescent protein (EGFP) expression in engineered mouse embryonic stem cells (mESCs) under selected human promoters of tissue-specific differentiation marker genes, such as Tubb3, VEGFR2, and Oct4. A proliferation study can be used to compare different culture conditions in that we can decide the optimal conditions for cells to grow. In my research, protocols for stem cell differentiation and proliferation were optimized for use in establishing a high throughput embryotoxicity screening platform, which can predict the embryotoxic potential of tested chemicals based on the green fluorescence from EGFP expression in mESCs in cell cultures. The optimized culture protocol is critical to improving mESC differentiation efficiency and should enhance the reproducibility and accuracy of the embryotoxicity screening platform.

Acknowledgments

I would like to thank my advisor Professor Shang-Tian Yang for giving me the opportunity for my honor research and supervising this thesis. It has been a privilege to work with him. I would like to thank my research mentor, Fengli Zhang, for guiding and helping me to become an independent researcher as well as suggestions for my career in research. Thanks to their patient guidance and support that I am able to finish this thesis.

I would like to acknowledge Professor Jessica Winter for joining my thesis committee, as well helping in my CBE courses.

To my family and friends, I would like to thank them for their encouragement and support especially Emilie Yeoh. This would so much harder without their help.

I would like to appreciate College of Engineering and University Honor Program for financial support and suggestions.

Lastly, I would like to thank all the members in Yang's lab during my research. Everyone was kind and helpful when I met troubles or had questions.

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Chapter 1: Introduction

Many chemicals used in food, drug and industrial products have not been assessed for their embryotoxicity, which can affect embryonic stem cell differentiation and cause changes in the development of embryo and result in malformation. Many studies have shown that exposure to embryotoxic chemicals such as organic solvent during pregnancy would cause low intrauterine growth rate and low birth weight [1]. What's more, using certain drugs during pregnancy can cause birth defects. For example, thalidomide caused the phocomelia of children in the 1960s [2]. It is thus important to screen chemicals for embryotoxicity before their uses in drugs, cosmetics, food, etc. and release to environment.

1.1 Stem cells

There are three kinds of stem cells in general: adult stem cells (ASCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs). ASCs are stem cells found in the human body, but most of them have specific tasks like hematopoietic stem cells, which can only become blood cells instead of other tissues. Thus, the application is limited. When women donate their eggs for research purposes, researchers can fertilize the eggs in vitro to form embryos and derive the inner cell mass into ESCs. ESCs have more possibilities than ASCs since ESCs can differentiate into all three

germ layers: ectoderm, endoderm, and mesoderm. However, there's some ethical issues associated with human embryonic stem cells. To get the inner cell mass, the embryo will be destroyed. In some states, the embryos are considered as a life that should be respected. Thus, many people think it's unethical to kill a life to get the human embryonic stem cells. Induced pluripotent stem cells are the medium in between adult stem cells and embryonic stem cells. Researchers can insert embryonic genes into somatic cells to make the somatic cells into "stem cells". Although this method has fewer ethical issues, developments are needed in ensuring the pluripotency and production iPSCs [3].

IPS techniques are capable of transferring any somatic cells into ES-like cells, but it has the risk of tumor formation since viral vectors are used to introduce embryonic genes [4]. Since there are some ethical issues in using human embryonic cells (hESCs) because deriving hESCs will destroy the embryo [4], it's a good idea to use mouse embryonic cells. Human and mouse embryonic stem cells are similar in the expression of many gene markers, even though humans and mice are different species [5].

1.2 Embryotoxicity testing

To test embryotoxicity, animal models are commonly used according to the Organization for Economic Cooperation and Development guidelines "prenatal developmental toxicity study" and "reproductive/developmentally toxicity screening test". Rabbits, mice, rats, and monkeys are most commonly used in research to determine LD50, which refers to the median lethal dose [6]. However, it is time-consuming and complex to do animal experiments, and a large number of sacrifices may be needed [7]. At least half of the testing animals will be killed to find the LD50 of certain drugs,

and thus, substitute methods are required to test the toxicity to reduce the cost and mortality. There are three alternatives in vitro methods to animal testing validated by the European Center for the Validation of Alternative Methods (ECVAM): whole embryo culture (WEC), mouse embryonic stem cell test (mEST), and the micromass (MM) test [8]. The micromass test is testing the chemical in the growing embryo at normal differentiation [9]. The whole embryo culture uses rat embryos that are around ten days of gestation and the visceral yolk sac from the embryo [10]. However, these two methods still need pregnant animals and result in sacrifices. Among them, the EST using mouse embryonic stem cells (mESCs) D3 and 3T3 fibroblasts is the only one implemented without sacrificing pregnant animals[11].

ESCs, derived from the inner cell mass in early embryonic development stages, are highly pluripotent and capable of differentiating into all three germ layers and various cell lineages [12], and are increasingly used as a novel tool in developmental risk assessment of environmental toxicants. Three endpoints are evaluated in EST and used to predict the embryotoxic potential (strong, weak, or none) of chemicals [13].

Current EST focuses on only one cell lineage, i.e. cardiomyocyte, because the heart is the first formed organ during organogenesis. EST takes 10 days and requires visual assessment of cardiomyocyte differentiation, which is not only time-consuming but also laborious in determining the endpoints [14]. Moreover, current EST cannot evaluate drug effects on stem cell differentiation to other cell lineages and has a $<80\%$ predictability in the validation studies [15].

Recently, our research group has developed a high throughput (HT) embryotoxicity screening based on mESCs expressing enhanced green fluorescent protein (EGFP)

driven by a human survivin promoter and cytomegalovirus (CMV) promoter, respectively [16]. The HT screening can monitor EGFP fluorescence as cell response to chemicals in real time with the engineered mESCs cultured in three-dimensional fibrous scaffolds in micro-bioreactors. This HT screening used survivin as the molecular endpoint and was validated with 9 chemicals with known embryotoxicity.

1.3 Scope of study

Chapter 2 presents proliferation study for comparing the effect of FBS from different vendors, effect of gelatin coating, and the growth curve by EGFP and AlamarBlue. This chapter is the basis of the experiments that we can know which way is better for the ES cell culture, so the conditions for the assay can be optimized. Proliferation study also validates the platform of high-throughput screening of EGFP.

Chapter 3 investigated cell differentiation experiments. Besides cardiomyocyte lineage, we also investigated neural cells since the nervous is essential in cell communication and respond to the outside environment. Process of optimization as well as the analysis are included in this this section. The optimized protocol can be used in the HT screening to achieve reliable results.

1.4 Significance

There are more than 10,000 industrial and emerging chemicals, including many used in pharmaceuticals, foods, cosmetics, plastics, and pesticides, waiting for embryotoxicity assessments, which will cost billion dollars and more than 30 years to complete if using animal models. Current EST for embryotoxicity screening does not sacrifice animal but is limited to only one cell lineage. A multi-lineage differentiation

strategy with three different molecular marker gene promoters can show how embryotoxic chemical may affect (inhibit) ESC differentiation to three different germ layers or cell lineages. This will overcome the drawbacks of current EST and provide a more robust and predictive embryotoxicity screening.

1.4.1 Proliferation study

A proliferation study can be used to compare the effects of culture conditions such as with gelatin-coating or without coating. A proliferation study is also important when determining the effects of different fetal bovine serum (FBS) from different vendors and to decide which one is better. Alamar Blue can be used to test cell proliferation because when it is blue without fluorescence, but it will be reduced to pink color and has fluorescence after incubation with cells. Compared to counting cell numbers manually, this method is quick and easy to perform.

1.4.2 Cell differentiation

My research focused on the development (optimization) of ESC differentiation protocols for use in the HT embryotoxicity screening. ESC growth and differentiation depends on many factors like temperature, coating, medium, etc. Wrong culture conditions and procedures would lead to cell death and the failure of the screening. The protocols available in the literature only contain rough culture steps and must be refined and optimized for our HT screening system. Optimization of culture protocols for ESC growth and differentiation is critical to the development of the HT screening and will help achieve reliable and reproducible results. Through finding the best way to perform each culture step, chemicals that may have effects on ESC growth and differentiation can be identified and assessed for their embryotoxic potential.

Chapter 2: Proliferation Study

2.1 Proliferation study to compare FBS

Proliferation study was performed to compare different fetal bovine serum (FBS) from different vendors: Sigma FBS, ES FBS, and HI FBS. FBS has growth factors, hormones, proteins and vitamins, which are important for cell proliferation [17]. ES FBS refers to embryonic stem-cell FBS from Gibco which is optimal for embryonic stem cells, and it's also the most expensive one. HI FBS refers to heat-inactivated FBS and it's also from Gibco. Heat-inactivated serum is using heat to inactivate some complements in the serum and is usually used when working with immune type cell cultures. Sigma FBS refers to FBS from Sigma-Aldrich, which has similar price compared to HI FBS. For each experiment, 10 % of FBS was added into the medium, for example, 4 ml FBS in 40 ml medium.

AlamarBlue was used to test the fluorescence of cells at different days. In this experiment, Wild type was used to compare the general effects of these 3 FBS. 10 % Alamarblue medium was used to test the fluorescence, for example, 1 ml of Alamar-Blue medium has 0.1 ml AlamarBlue with 0.9 ml of medium. AlamarBlue was tested at exitation wavelength of 535 nm and emission wavelength of 595 nm. After testing

Table 2.1: Experimental design for comparing FBS

Sigma FBS	Sigma FBS	Sigma FBS	Control
HI FBS	HI FBS	HI FBS	Control
ES FBS	ES FBS	ES FBS	Control

the fluorescence, we can plot the growth curve of cells under each FBS to see the effects of different FBS. Higher fluorescence values means more AlamarBlue is reduced, and thus it means there are more cells. For each test, new AlamarBlue medium is needed. Normal growth medium needs to be replaced by AlamarBlue medium, and then after testing the fluorescence values in 3 hours, the reduced AlamarBlue medium needs to be replaced by normal growth medium. For each well, we put 20 thousand cells and 0.25 ml medium. Initially, for the first trial, we decided to use 0.2 ml of medium + Alamar blue, however, there were too many cells, so there was no reading of fluorescence after incubation for 3 hours since the values exceeded the maximum value of the testing machine. For the second trial, we changed the medium + Alamar Blue amount to 0.8 ml, and this amount of medium worked well. Table 2.1 is the experimental design. 3 repeated wells on 24-well plate were used to lower the effect of random errors, and one control well for each FBS was used.

From Figure 2.1, we can see there is no significant difference in cultures growing in these 3 FBS.

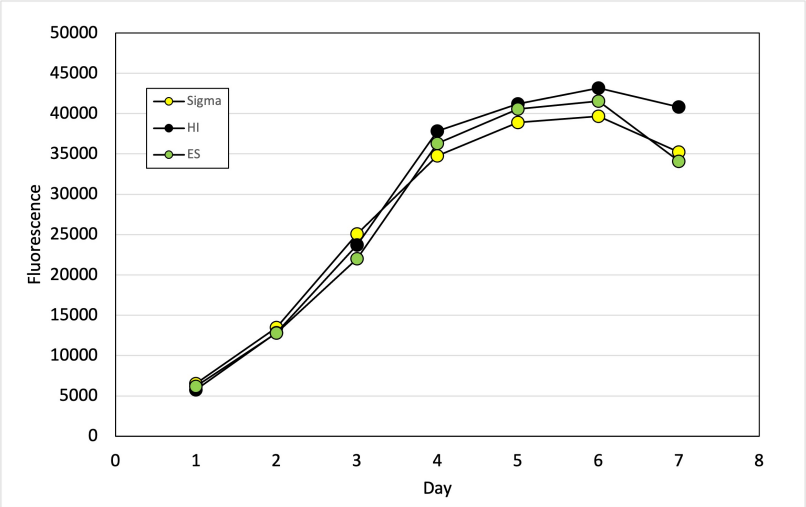


Figure 2.1: Fluorescence of different FBS

2.2 Growth curves by EGFP and AlamarBlue

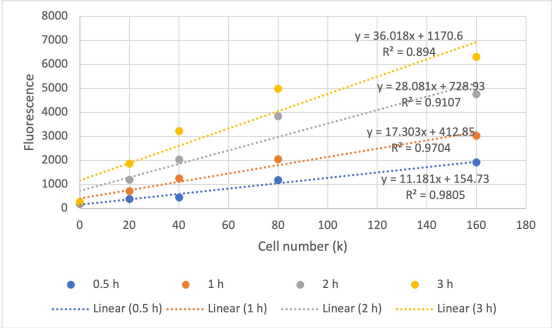
2.2.1 Pre-experiment

96-well plate was used in this pre-experiment. We used four wells to mimic cell number for four different days, and upper wells using 0.2 ml and lower wells using 0.25 ml medium. EGFP was tested 2 hours after seeding the cells at exitation wavelength of 485 nm and emission wavelength of 535 nm. 10 % AlamarBlue medium was used, and Alamar blue fluorescence was test at half an hour, 1 hour, 2 hours, and 3 hours to compare the effects of incubation time. Instead of 0.8 ml of 10 % AlamarBlue medium, only maximum of 0.25 ml medium can be added into each well of 96-well plate, thus it's important to determine the amount of medium and incubation time before actual experiments. Table 2.2 shows the experimental design of this pre-experiment. for upper 5 wells, 0.2 ml was used and for lower 5 wells, 0.25 ml of medium was used, and gain values of 25 and 35 were used to test the fluorescence. Four wells were used

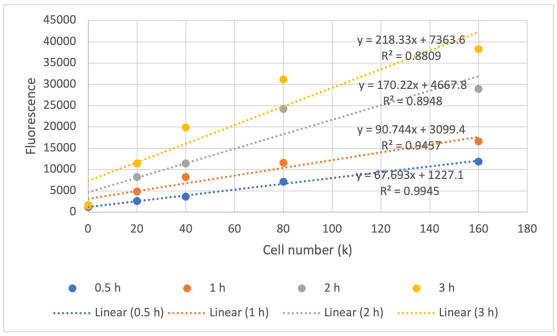
to mimic cells in four days, each well is double the number of cells of the previous well.

Table 2.2: Experimental design for the pre-experiment

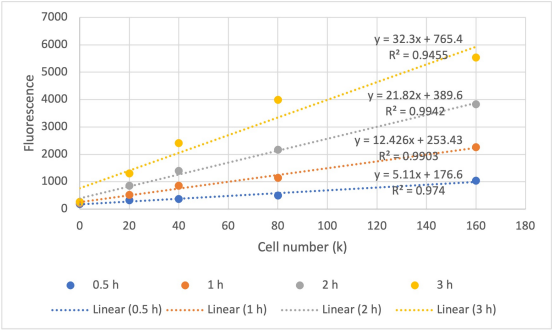
20,000 cells	40,000 cells	80,000 cells	160,000 cells	Control (Only 0.2ml medium)
20,000 cells	40,000 cells	80,000 cells	160,000 cells	Control (Only 0.25ml medium)



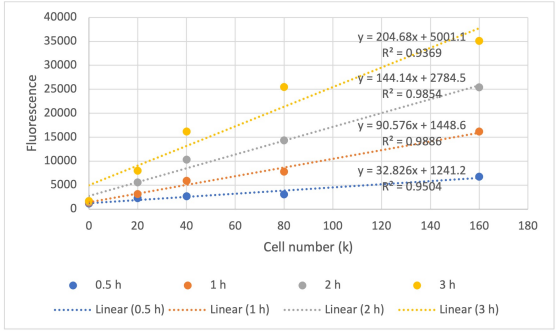
(a) 0.2 ml; Gain: 25



(b) 0.25 ml; Gain: 35



(c) 0.25 ml; Gain: 25



(d) 0.25 ml; Gain: 35

Figure 2.2: AlamarBlue fluorescence at different medium volume and gain

From Figure 2.2, we can see that amount of medium doesn't have a significant effects on alamar blue fluorescence, and 1 hour is the best incubation time. The yellow

dots and trendlines refer to 3 hours incubation, and we can see that the relationship is not linear anymore, which means there's not enough alamar blue that can be reduced.

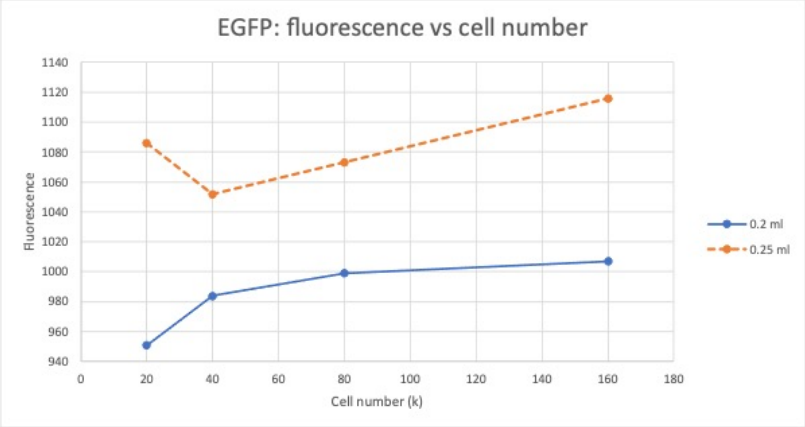


Figure 2.3: EGFP fluorescence at different medium volume

From figure 2.3 we can see that the orange curve which refers to 0.25 ml medium, has an abnormal trend while fluorescence value should increase when cell number increases. Considering material usage and error, we decided to use the combination of 1 hour and 0.2 ml of medium.

2.2.2 First trial

In the first trial of experiment, we decided to use ES-Tubb3-EGFP and ES-CMV-EGFP cell lines, so that we can exclude the effect of specific cell lines. Each cell line has 5 wells to lower the chance of errors, and 1 well of control which is same amount of medium without cells. EGFP and AlamarBlue were tested once a day and AlamarBlue was tested 1 hour after EGFP.

Table 2.3: Experimental design for trial 1

Tubb3	20 k	20 k	20 k	20 k	20 k	1 Control
CMV	20 k	20 k	20 k	20 k	20 k	1 Control
Wild type	20 k	20 k	20 k	20 k	20 k	1 Control

However, the result shows that the measurements were too less to have a complete curve. What's more, there may be some random error in measuring fluorescence, so 1 control is not enough to lower the effect of random error. Thus, we decided to use 3 controls in the next trial.

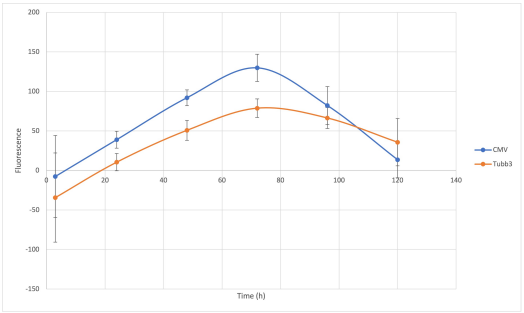


Figure 2.4: EGFP growth curve

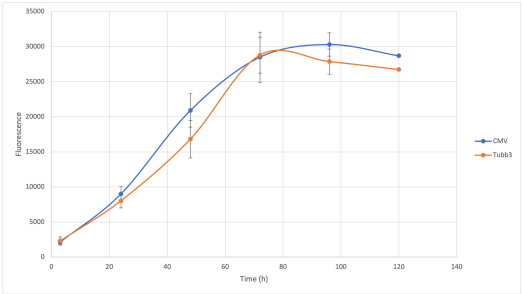


Figure 2.5: AlamarBlue growth curve

2.2.3 Second trial

In the second trial, we used the same conditions as trials 1, the only difference is we decided to measure fluorescence twice or third times a day depending on cell conditions. Furthermore, we added one more cell line of ES-wild type to provide a comparison of EGFP for engineered cells and non-engineered cells.

Table 2.4: Experimental design for second trial

Tubb3	20 k	20 k	20 k	20 k	20 k	3 Controls
CMV	20 k	20 k	20 k	20 k	20 k	3 Controls
Wild type	20 k	20 k	20 k	20 k	20 k	3 Controls

Since the same wells of cells were used to measure both EGFP and AlamarBlue, the fluorescence of EGFP was greatly affected by changing medium too often.

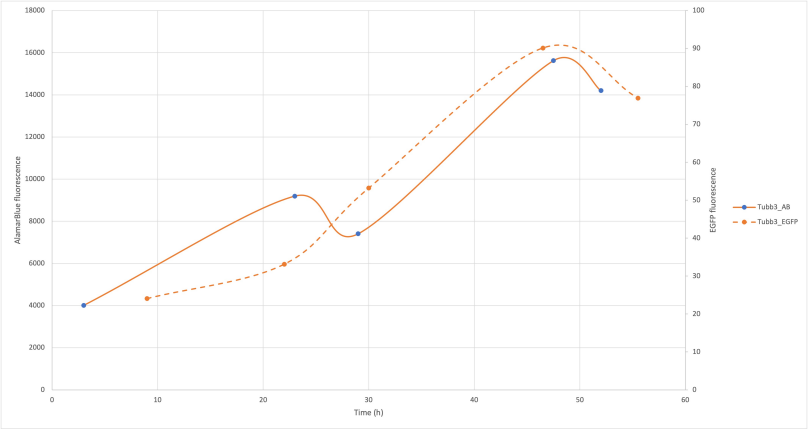


Figure 2.6: Tubb3 growth curves by EGFP and AlamarBlue

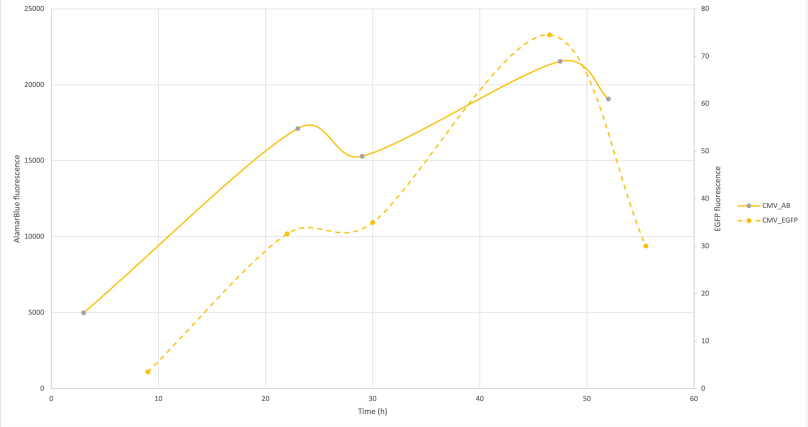


Figure 2.7: CMV growth curves by EGFP and AlamarBlue

2.2.4 Third trial

In the last trial, we changed the experimental design that two sets of different wells were used for EGFP and AlamarBlue seperately to avoid the effect of medium change. 3 controls were used to avoid random measurement errors.

Table 2.5: Experimental design for third trial

EGFP	Tubb3	20 k	20 k	20 k	20 k	20 k	3 Controls
EGFP	CMV	20 k	20 k	20 k	20 k	20 k	3 Controls
EGFP	Wild type	20 k	20 k	20 k	20 k	20 k	3 Controls
AB	Tubb3	20 k	20 k	20 k	20 k	20 k	3 Controls
AB	CMV	20 k	20 k	20 k	20 k	20 k	3 Controls
AB	Wild type	20 k	20 k	20 k	20 k	20 k	3 Controls

From Figure 2.8 and 2.9 we can see that the trends of growth curves by EGFP and AlamarBlue are similar for Tubb3 and CMV cell lines. However, for Wild type in Figure 2.10, the growth curve by EGFP plateaus at time around 40 hours, and then

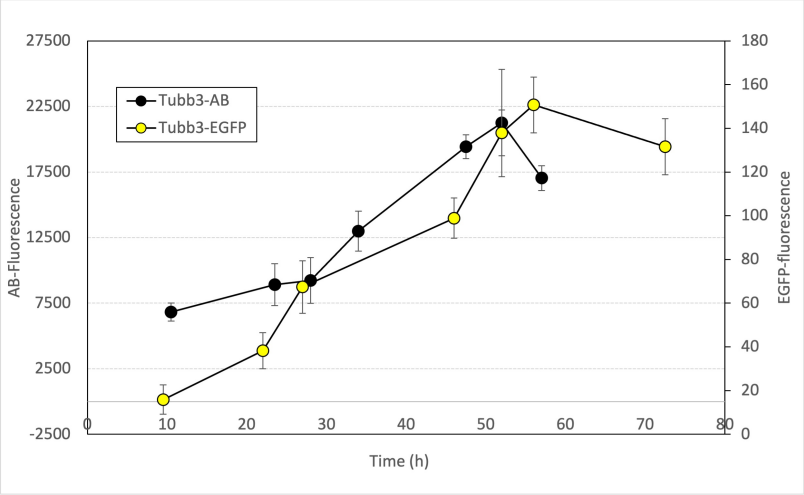


Figure 2.8: Tubb3 growth curve by EGFP and AlamarBlue

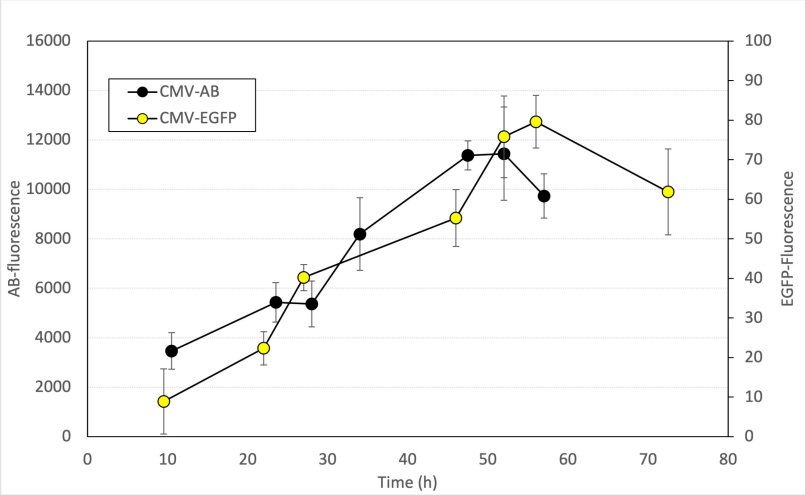


Figure 2.9: CMV growth curve by EGFP and AlamarBlue

the curve increased rapidly after certain point. We assumed that when cell number reaches certain point, the cells will have fluorescence by themselves instead by EGFP genes at the EGFP wavelength range. This can also proves EGFP can't be used for

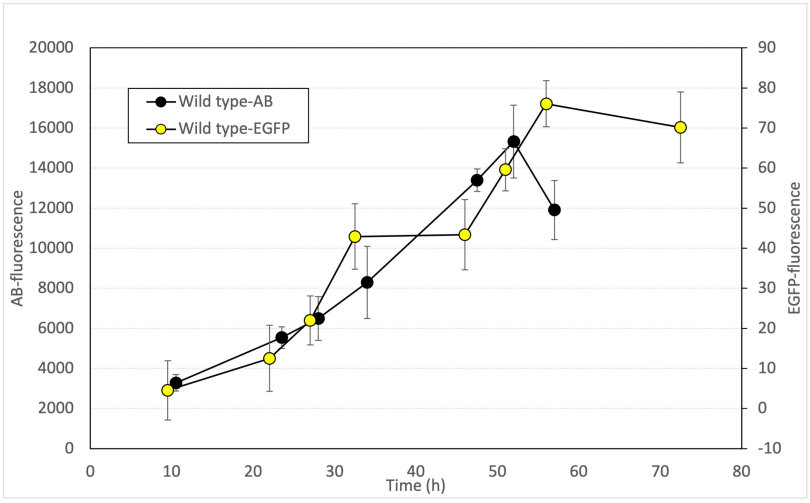


Figure 2.10: Wild type growth curve by EGFP and AlamarBlue

Wild type cell line since it doesn't have the EGFP gene. This trend shows that EGFP is good in modeling cell conditions and thus it's good in predicting embryotoxicity.

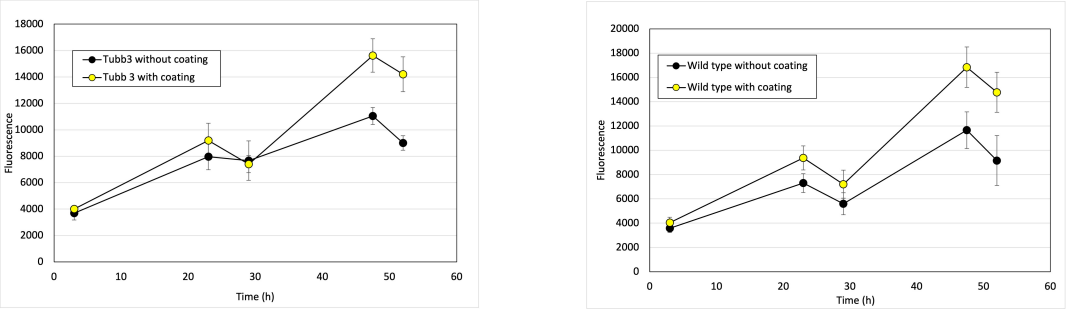
2.3 Proliferation Study for effect of gelatin coating

Proliferation study was also used to test the effect of gelatin coating. The cells growing in the plates with coating and without coating were similar when observing under microscope. Thus, proliferation study was performed to evaluate the effect of gelatin coating to see if it's necessary. Table 2.6 is the experimental design for comparing the effects of gelatin coating. 20 thousands cells were seeded into 1 well of 96-well plate, and 0.2 ml medium was used for each well.

Figure 2.11 shows that the fluorescence values are similar initially, however, after several days, the fluorescence values deviate. Fluorescence values of gelatin coating are getting higher than values of without gelatin coating. From this result, we can

Table 2.6: Experimental design for effects of gelatin coating

Coat	Tubb3	20 k	20 k	20 k	20 k	20 k	Control
Without	Tubb3	20 k	20 k	20 k	20 k	20 k	Control
Coat	Wild type	20 k	20 k	20 k	20 k	20 k	Control
Without	Wild type	20 k	20 k	20 k	20 k	20 k	Control



(a) Tubb3

(b) Wild type

Figure 2.11: Comparison of gelatin coating

conclude that gelatin coating is good for cell culture that cells can have a better condition to grow. The points for 29 h are off due to errors when measuring fluorescence. Despite the error, we can still see that the fluorescence values of gelatin coating wells are higher than the values of without gelatin coating.

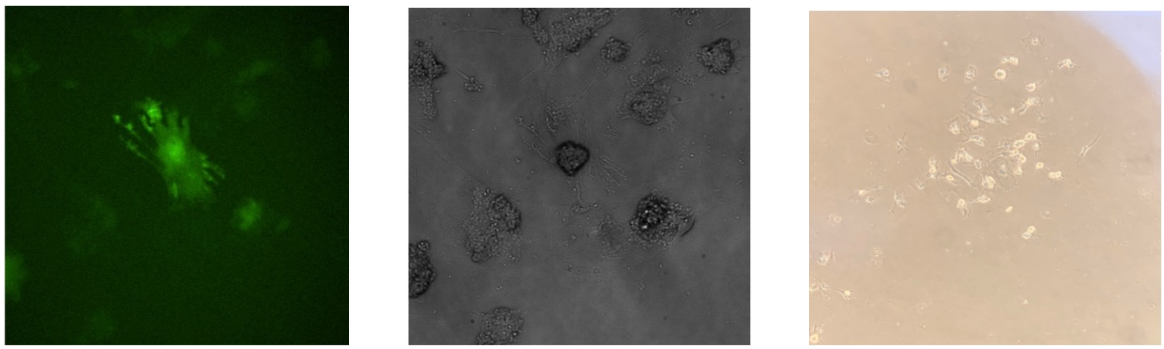
Chapter 3: Cell differentiation

3.1 Neural differentiation

Three different culture media are used for studying ESC neural differentiation. Medium 1, Dulbecco's Modified Eagle Medium (DMEM) containing 1 % non-essential amino acid, 1% penicillin-streptomycin, 0.5% L-glutamine, 0.2% β-mercaptoethanol, 10% fetal bovine serum (FBS), and 0.1% Leukemia inhibitory factor (LIF), is used to grow ES cells and keep their pluripotency without spontaneous differentiation. The medium with the same composition but without LIF (Medium 2) is used to start cell differentiation, while Medium 2 with 0.1 % of 10^{-4} M retinoic acid for neural differentiation (Medium 3).

The 4-/4+ protocol was used for ES-Tubb3-EGFP differentiation into neuronal cells (Zang & Yang, 2013). Retinoic acid of 10-2 M is used for neural differentiation and diluted into 10-4 M for culture medium. ES-Tubb3-EGFP cells are grown in 6-well plate for 3 days with Medium 1 and on day 4, cells are transferred onto 2 non-adherent 9 cm petri dish with Medium 2 and incubated on an orbital shaker for 4 days. On day 8, retinoic acid is added into Medium 2 and cells are incubated on an orbital shaker for another 4 days. On day 12, 50 embryo bodies (EBs) are picked into each well in 6-well plates from one of the petri dishes without adding medium

and replace the medium in dish 2 with Medium 2. As can be seen in Figure 3.1, long neural axons appeared after day 18.

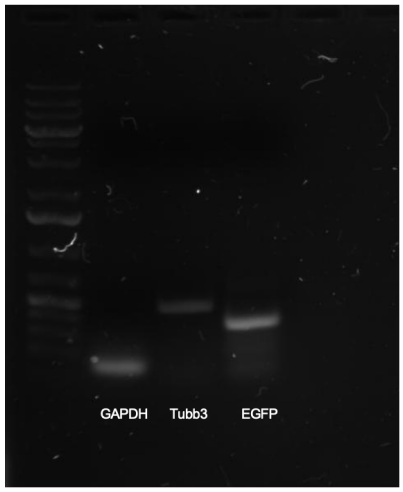


(a) EGFP expression (b) Under bright field (c) Under microscope

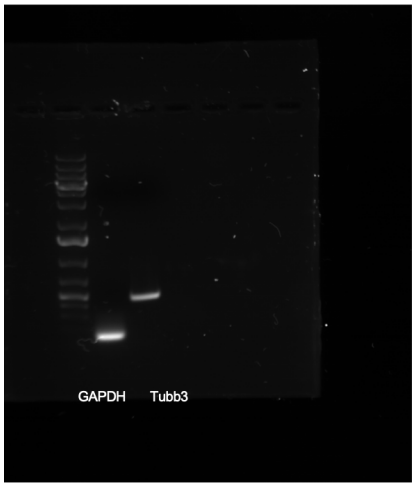
Figure 3.1: Differentiated neural cells

RT-PCR was then performed on day 27 and it showed Tubb3 (a neural differentiation marker) was highly expressed. Green fluorescence was also detected, which clearly illustrated the morphology of differentiated neural cells. The green fluorescence in the culture can be readily monitored with a spectrofluorometer and used as indicative for the degree of differentiation, which in turn can be used to assess drug effect on ESC differentiation. Due to too less number of differentiated cells, we were not able to test EGFP gene expression of differentiated cell. From Figure 3.2 and 3.3, we can see that the gene expression of Tubb3 is much higher after differentiation compared to undifferentiated cells, while we still need to test EGFP gene of differentiated cells to finish the comparison.

However, the cell adhesion on 6-well plate was not good. We waited for 24 hours after picking EBs into 6-well plate, but the cell aggregates probably were not in a good health condition. After adding enough amount of medium and observed for few



(a) Gene expression before differentiation



(b) Gene expression after differentiation

Figure 3.2: RT-PCR result

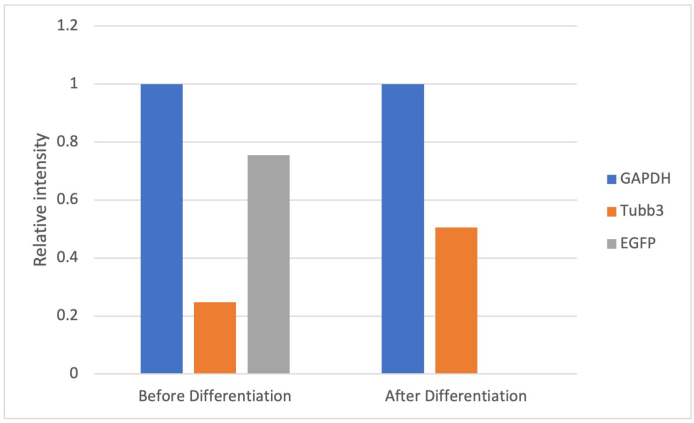


Figure 3.3: ES-Tubb3-EGFP neural differentiation

days, none of the cell aggregates were growing. The factors that may be involved in cell adhesion include: amount medium added initially, Whether to move cells to new environment or not, Size of original cells aggregates, Whether to remove all the medium at once and replace with new medium. Thus we decided to shorten the time

of adding enough medium. Throughout the experiment, we noticed that it's better not to change medium often when it's on orbital shaker, which means small amount of cells would be better. When changing medium, we kept the dish in the hood and allowed EBs to settle to bottom, then removed the supernatant from the dish and replaced it with 10 ml fresh medium 2.

3.2 Cardiac differentiation

Three different culture media are used for studying ESC cardiac differentiation, respectively. Medium 1, Dulbecco's Modified Eagle Medium (DMEM) containing 1 % non-essential amino acid, 1% penicillin-streptomycin, 0.5% L-glutamine, 0.2% β-mercaptoethanol, 10% fetal bovine serum (FBS), and 0.1% Leukemia inhibitory factor (LIF), is used to grow ES cells and keep their pluripotency without spontaneous differentiation. The medium with the same composition but without LIF (Medium 2) is used to start cell differentiation, while Medium 2 with 20 % of FBS for cardiac differentiation (Medium 3).

3.2.1 First trial

For the first trial, the ES-wild type and cardiomyocyte differentiation protocol optimized by Hartman et al. (2014) was used. ESC cells grew in Medium 1 for 4 days and the hanging drop method was used in day 5 to create aggregates in Medium 3. For this method, we added 70 drops on each lid of the petri dish with one drop containing 20 μL medium and 1000 cells like shown in Figure 3.4, and then the dishes were filled with 7 ml phosphate-buffered saline (PBS). After 3 days, the aggregates were transferred into another petri dish and Medium 3 was added as the differentiation medium. After 4 days, each EB was transferred into a 24 well plate and observes the

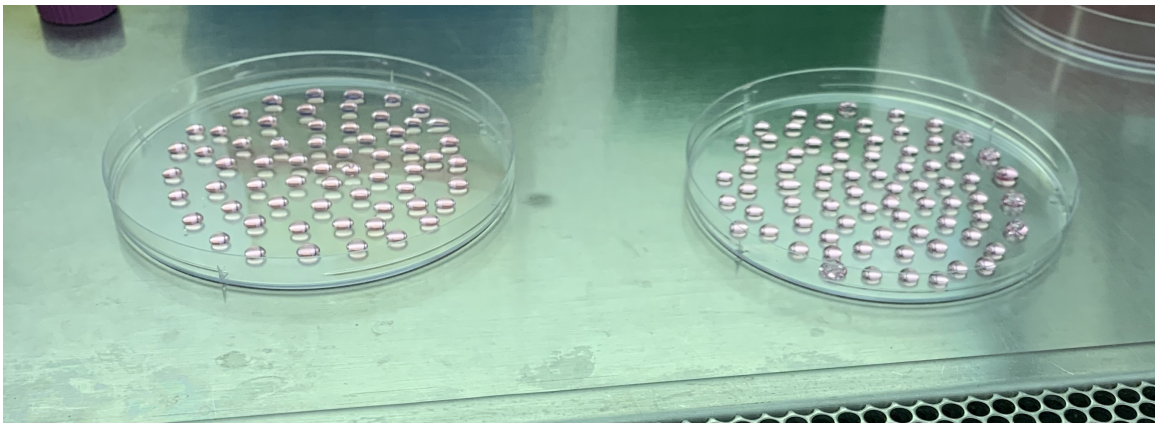


Figure 3.4: Hanging drop method

beating of EBs. No beating cells in any of the wells or dishes were observed under microscope. In this trial, we observed that when we put EBs in petri dishes, they will adhere to the bottom of the dishes instead of floating in the medium. Thus, it was hard to find a contact round EB and transfer to the 24-well plate. Hanging drop method is good in controlling the size of the cell aggregates, but it takes time to perform and it's hard to take the cell aggregates out from the lids of the dishes. Considering the adhesion of cell aggregates and complexity, we decided to use the orbital shaker method in the second trial.

3.2.2 Second trial

In the second trial, we used ES-VEGFR2-EGFP cell line. The gene expression of VEGFR2 will increase when the cells are differentiated into cardiac cells, so that RT-PCR can be performed to test the cell differentiation. However, during the experiments, this cell line got contaminated and failed to have any result. Thus we changed to ES-OCT4-EGFP cell line.

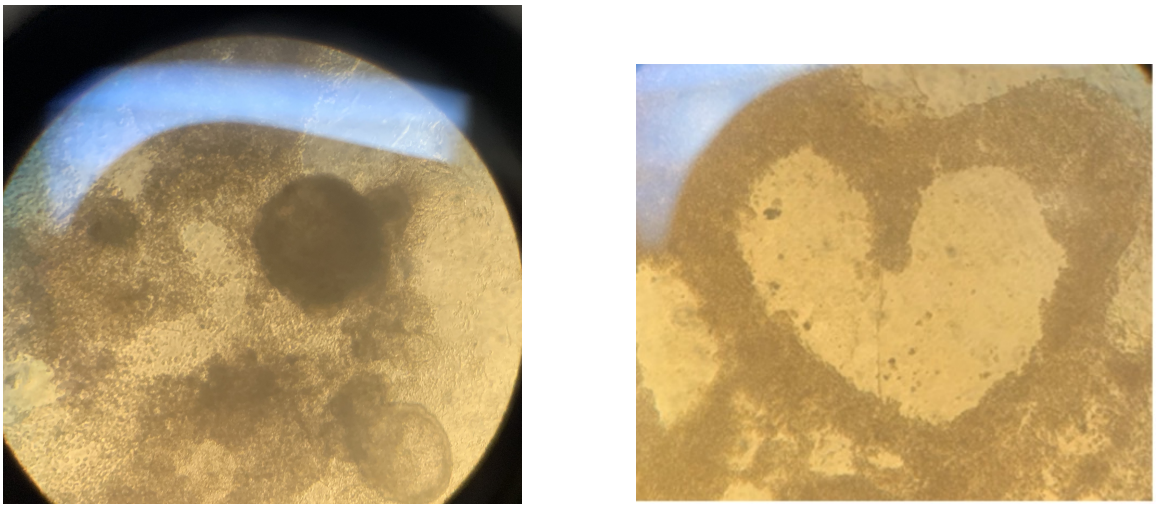
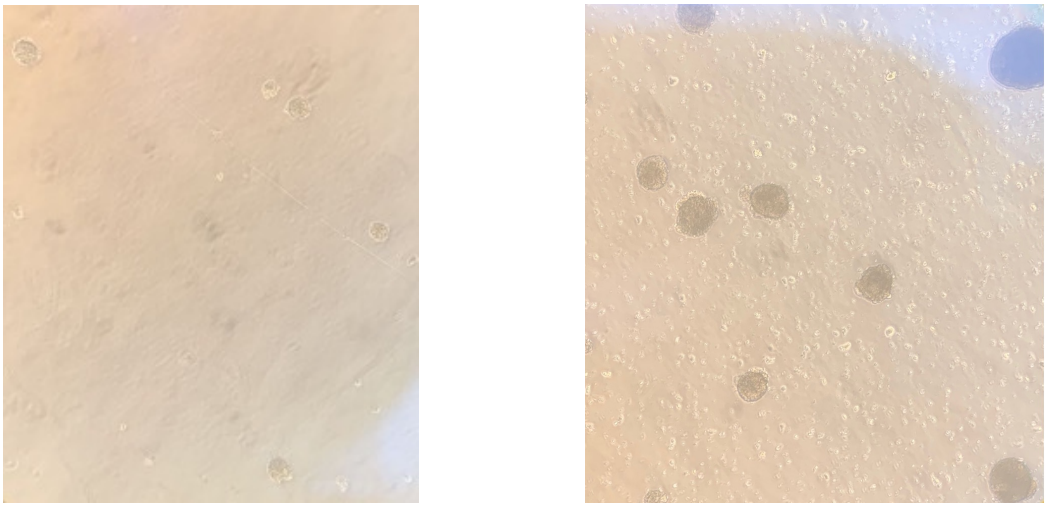


Figure 3.5: Cell adhesion on dish

3.2.3 Third trial

Optimized protocol and ES-OCT4-EGFP were used in the third trial. Cells were growing in medium 1 for 4 days, and on day 5, 50 k cells were seeded into each dish in medium 2 and put on the orbital shaker for 3 days. On the next day, small aggregates were found and cell aggregates became larger on the following days. When changing medium, let the cell aggregates settle down for 5 min, remove the supernatant as much as possible without picking any cell aggregates. On day 8, medium 3 was prepared, and medium 2 is replaced by medium 3. In this stage, cell aggregates were still getting larger. On day 12, EBs were transferred into 24-well plate. One EB was picked and seeded into one well of 24-well plate. For the upper 12 well, 50 μL medium was added in each well, and for the lower 12 well, 100 μL medium was added in each well. 0.5 ml of medium was added to each well the next day in 16 hours to ensure cells' proliferation. This amount of time ensured cell aggregates adhesion to

plates and dishes as well as cell proliferation. Cells were growing after adhered to plates or dishes but no beating was observed. RT-PCR was performed to test the gene expression, but due to technical issues, there was no result.

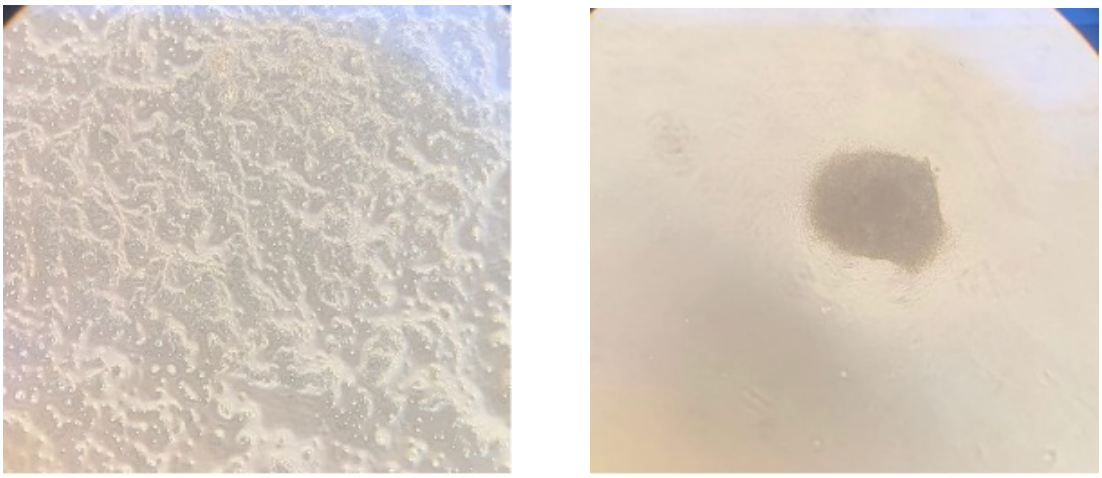


(a) Cell aggregates on day 2

(b) Cell aggregates on day 4

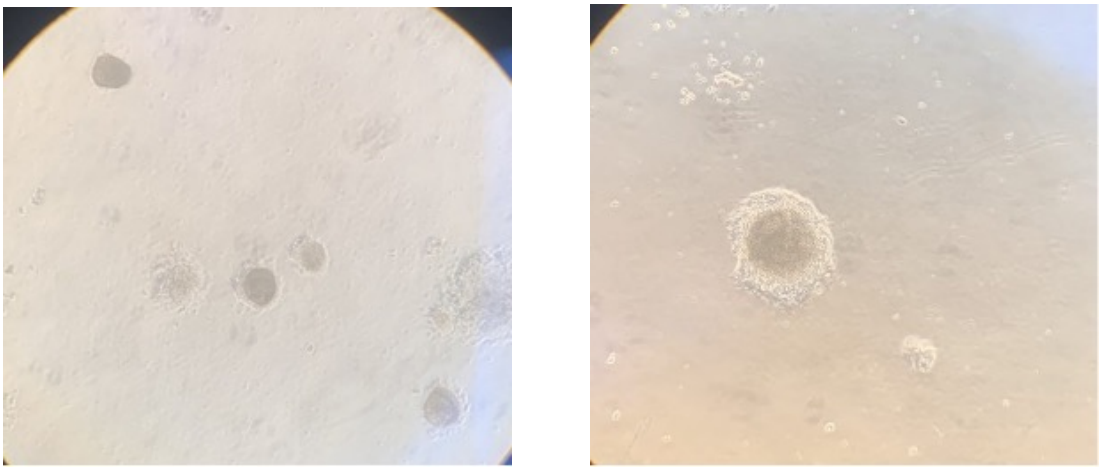
Figure 3.6: Cell aggregates

Cell aggregates were observed the next day on orbital shaker. The shapes of cell aggregates were rounder and more transparent compared to neural differentiation, and thus this cell aggregates adhered well on both petri dishes and 24-well plates. From Figure 3.7a, we can see that when transferring cell aggregates, it was hard to keep the aggregates intact, so the cell debris may be every where on the well of the plate. Also, from Figure 3.7b, we can see that even if the EB adhered to the plate, the adhesion was not good. From Figure 3.8a and 3.8b, the morphology on petri dishes were better compared to cell adhesion on 24-well plate. This result implies dish may be a better choice for cell adhesion and differentiation.



(a) Well 1 (b) Well 2

Figure 3.7: Cell adhesion on 24-well plate



(a) Dish 1 (b) Dish 2

Figure 3.8: Cell adhesion on Petri dishes

Chapter 4: Conclusion

This thesis presents the optimization of cell differentiation protocols and proliferation study. Chapter 2 presents the improvements in methods and results in proliferation study. This chapter shows that there was no significant difference in these 3 FBS that we chose for experiments, and this experiment also implies that we can use a proliferation study to compare the difference between different reagents. Comparing the effect of gelatin coating implies that although the morphology of cells may be similar under a microscope, the long-term growth may be affected since the fluorescence values deviated more when time increases. Thus it's important to use proliferation study to find optimal conditions for cells to grow since it's hard to determine based on morphology. Growth curves by EGFP and AlamarBlue prove that trends of fluorescence measured using EGFP were similar to trends of AlamarBlue fluorescence Thus, it's a reliable way to determine cell proliferation using EGFP.

In Chapter 3, optimization of cell differentiation is presented. Orbital shaker is definitely a better method in creating cell aggregates since it's easy to perform and efficient. One challenge in this work is that there are too many factors that can affect cell conditions, so it's hard to determine what is the real cause of the failures of the experiments. Thus, the optimized protocols should be easy to perform and have a high tolerance of random errors.

Future works include RT-PCR of neural differentiation and cardiac differentiation. RT-PCR should be performed to test the gene expressions of differentiated cells to verify the feasibility of the optimized protocols.

Bibliography

[1] Parvez Ahmed and Jouni JK Jaakkola. Exposure to organic solvents and adverse pregnancy outcomes. *Human reproduction*, 22(10):2751–2757, 2007.

[2] Punam Sachdeva, BG Patel, and BK Patel. Drug use in pregnancy; a point to ponder! *Indian journal of pharmaceutical sciences*, 71(1):1, 2009.

[3] University of nebraska medical center. Educational resources: stem cells. `https://www.unmc.edu/stemcells/educational-resources/`.

[4] Catherine H Hackett and Lisa A Fortier. Embryonic stem cells and ips cells: sources and characteristics. *Veterinary Clinics: Equine Practice*, 27(2):233–242, 2011.

[5] Irene Ginis, Yongquan Luo, Takumi Miura, Scott Thies, Ralph Brandenberger, Sharon Gerecht-Nir, Michal Amit, Ahmet Hoke, Melissa K Carpenter, Joseph Itskovitz-Eldor, et al. Differences between human and mouse embryonic stem cells. *Developmental biology*, 269(2):360–380, 2004.

[6] FD Griffith and JE Long. Animal toxicity studies with ammonium perfluorooctanoate. *American Industrial Hygiene Association Journal*, 41(8):576–583, 1980.

[7] Esther de Jong, Marta Barenys, Sanne AB Hermsen, Aart Verhoef, Bernadette C Ossendorp, Jos GM Bessems, and Aldert H Piersma. Comparison of the mouse embryonic stem cell test, the rat whole embryo culture and the zebrafish embryotoxicity test as alternative methods for developmental toxicity testing of six 1, 2, 4-triazoles. *Toxicology and applied pharmacology*, 253(2):103–111, 2011.

[8] Elke Genschow, Horst Spielmann, Gabriele Scholz, Andrea Seiler, Nigel Brown, Aldert Piersma, Madeleine Brady, Nicole Clemann, Hannele Huuskonen, Francoise Paillard, et al. The ecvam international validation study on in vitro embryotoxicity tests: results of the definitive phase and evaluation of prediction models. *Alternatives to Laboratory Animals*, 30(2):151–176, 2002.

[9] Oliver P Flint. In vitro tests for teratogens: desirable endpoints, test batteries and current status of the micromass teratogen test. *Reproductive Toxicology*, 7:103–111, 1993.

[10] Peng Zou, Lina Xing, Qiuqiong Tang, Weidong Hao, et al. Comparative evaluation of the teratogenicity of genistein and genistin using rat whole embryo culture and limbud micromass culture methods. *Food and chemical toxicology*, 50(8):2831–2836, 2012.

[11] H Spielmann, I Pohl, B Doring, M Liebsch, and F Moldenhauer. The embryonic stem cell test, an in vitro embryotoxicity test using two permanent mouse cell lines: 3t3 fibroblasts and embryonic stem cells. *In Vitro Toxicology*, 10(1):119–128, 1997.

[12] Richard A Young. Control of the embryonic stem cell state. *Cell*, 144(6):940–954, 2011.

[13] G Scholz, E Genschow, I Pohl, S Bremer, M Paparella, H Raabe, J Southee, and H Spielmann. Prevalidation of the embryonic stem cell test (est)—a new in vitro embryotoxicity test. *Toxicology in vitro*, 13(4-5):675–681, 1999.

[14] Noriyuki Suzuki, Satoshi Ando, Norihisa Yamashita, Nobuyuki Horie, and Koichi Saito. Evaluation of novel high-throughput embryonic stem cell tests with new molecular markers for screening embryotoxic chemicals in vitro. *Toxicological Sciences*, 124(2):460–471, 2011.

[15] Andrea EM Seiler and Horst Spielmann. The validated embryonic stem cell test to predict embryotoxicity in vitro. *Nature protocols*, 6(7):961–978, 2011.

[16] Ru Zang, Xin Xin, Fengli Zhang, Ding Li, and Shang-Tian Yang. An engineered mouse embryonic stem cell model with survivin as a molecular marker and egfp as the reporter for high throughput screening of embryotoxic chemicals in vitro. *Biotechnology and bioengineering*, 116(7):1656–1668, 2019.

[17] Jan van der Valk, Karen Bieback, Christiane Buta, Brett Cochrane, Wilhelm Dirks, Jianan Fu, James Hickman, Christiane Hohensee, Roman Kolar, Manfred Liebsch, et al. Fetal bovine serum (fbs): past–present–future. *Altex*, 35(1):1–20, 2018.